the complex. The positioning of a subunit in the complex may be crucial to its function. The dynamic equilibrium between various assembly states of a heteropolymer cannot be treated as if the assembly involves structurally homogeneous elements whose association reactions can be described by a single interaction constant. On the contrary, each stage of assembly can be assumed to have a definite probability of adding another subunit which depends upon both the state of aggregation and the stereochemistry of the subunit. Problems of "micro-heterogeneity" in associating systems have puzzled, and plagued, researchers for decades. Observations of micro-heterogeneity in oligomers may to some extent be a consequence of nonequivalence of subunit types. An understanding of the intermolecular forces which govern the dissociation behavior of the mollusc and arthropod hemocyanins may provide an insight into the assembly of other proteins where functionally and structurally diverse subunits are known to play critical roles in the aggregation phenomena.

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ASSEMBLY OF CATALYTIC SUBUNITS OF ASPARTATE TRANSCARBAMOYLASE FROM *ESCHERICHIA COLI*

Drusilla L. Burns and H. K. Schachman, *Department of Molecular Biology and Virus Laboratory, University of California, Berkeley, California 94720 U.S.A.*

Although extensive studies have been conducted on the assembly of the allosteric enzyme, aspartate transcarbamoylase (ATCase) from isolated, intact catalytic (*C*) and regulatory (*R*) subunits, there has been little research on the formation of these subunits from individual catalytic (*c*) and regulatory (*r*) polypeptide chains. Such studies would be useful for evaluating the strengths of the interchain bonding domains within the subunits just as earlier experiments provided valuable data regarding interactions between the subunits in ATCase. The intact enzyme comprising two *C* trimers and three *R* dimers is designated as C_2R_3 or c_6r_6.

Isolated *C* trimers, in contrast to intact ATCase, exhibit Michaelian kinetics and no inhibition by CTP (or activation by ATP). The trimers are very stable and no dissociation has been observed in the ultracentrifuge even at concentrations <2 μg/ml. However, hybrids were detected when mixtures of native (C_N) and succinylated (C_S) subunits were incubated at 0°C for several days. The dissociation (and hybridization) of the subunits was decreased markedly (Fig. 1) upon the addition of the bisubstrate analog, *N*-(phosphonacetyl)-L-

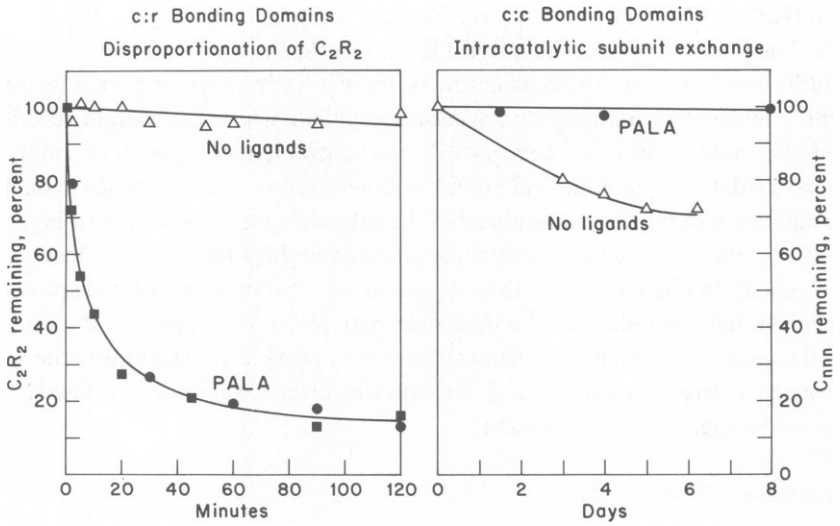

Figure 1 The effect of ligands on intersubunit bonds in ATCase. The figure on the left shows the effects of PALA on the disproportionation of C_2R_2. This disproportionation process, 3 $C_2R_2 \rightarrow 2\ C_2R_3 + 2C$, involves as a first reaction the rupture of *c:r*"bonds." The effect of PALA on the rate of intracatalytic subunit exchange is shown on the right. A 1:1 mixture of ^{125}I-labeled trimers (C_N or C_{nnn}) and succinylated trimers (C_S) was incubated in 0.04 M Tris-HCl, pH 8.0, at 0°C. An identical mixture contained 1.3 mM PALA. The percentage of counts remaining as C_{nnn} trimers was determined by sampling the mixtures at various times and separating the species by polyacrylamide gel electrophoresis. The gels were sliced and then counted on a Nuclear-Chicago gamma counter.

aspartate (PALA). Hence this active-site ligand causes a large increase in the strength of the three heterologous *c:c*"bonds" within the *C* subunits. In contrast, PALA causes a several hundred-fold weakening of the *c:r* bonding domains in ATCase-like molecules (Fig. 1) lacking one *R* subunit (C_2R_2). Moreover, these two types of"bonds"apparently have opposite temperature coefficients, with the *c:c* "bonds"becoming stronger and the *c:r*"bonds"weaker as the temperature is increased.

The *C* subunits can be dissociated into inactive monomers by various denaturants and then reconstituted in high yield by removal of the perturbants; hence the kinetics of regeneration of enzyme activity and the assembly of intermediates and final product can be measured.

Characterization of C Subunits in Urea and NaSCN

Sedimentation equilibrium experiments showed that monomers (M_r = 33,000) were formed in 4.7 M urea at 0°C or in 1.25 M NaSCN at 25°C. The urea-denatured species had an $s_{20,w}$ of 1.7 S and an f/f_0 of 2.15, whereas the corresponding values were 2.8 S and 1.27 for the monomers in NaSCN. Difference spectra of the protein in urea showed a marked perturbation of the aromatic side chains. The spectral change for the NaSCN-produced monomers was much smaller. These hydrodynamic and spectral studies indicate that the monomers in NaSCN were much more compact and structured than those in urea.

Reconstitution of the C Trimers from Urea and NaSCN

Assembly of the subunits was initiated by dilution of the dissociated protein into 0.04 M Tris-HCl buffer containing 2 mM mercaptoethanol at pH 7.5 and 0°C. The process was monitored at various times for (*a*) restoration of enzymic activity, (*b*) formation of trimers indicated by the lack of hybridization with c_S chains, and (*c*) formation of intermediates

detected by crosslinking the species with glutaraldehyde. For many of the experiments ^{125}I-labeled *C* subunits were used; thus,"stopping"the assembly by the addition of excess inactive C_S chains (produced by dissociation of C_S trimers) permitted us to determine how much protein had already been incorporated into C_N trimers before the "chase." The monomers present at the time of the addition of c_S were assembled into hybrids which migrated to different positions upon electrophoresis in polyacrylamide gels. Dimers were determined by electrophoresis experiments in gels containing sodium dodecyl sulfate; for these experiments the reconstitution was"stopped"by the addition of glutaraldehyde which crosslinked trimers efficiently and rapidly.

The half-time for reactivation of the urea-denatured species was 50 min at 0°C and was independent of concentration from 0.1 to 0.4 mg/ml. First-order kinetics was observed in all experiments. Moreover, electrophoretic determinations of the formation of C_N trimers in the experiments stopped with c_S showed that the rate of assembly of trimers was identical to that for the restoration of enzyme activity.

Reactivation of the NaSCN-dissociated protein was much more rapid than that for the urea-denatured protein. At a concentration of 0.37 mg/ml the half-time was 5 min and it increased as the protein concentration was lowered. Data from experiments over a limited concentration range fit second-order kinetics and the crosslinking experiments showed that the predominant species throughout the assembly process were trimers and monomers; the dimer concentration was less than 5% (of the total protein).

These results allow us to describe the events in the assembly of *C* subunits. Since the rate of reactivation of the urea-denatured species is identical to the rate of trimer formation, the rate-limiting step must be a folding of the individual chains which must occur before association can take place. In contrast, the half-time for reactivation of the NaSCN-denatured protein is concentration-dependent; hence the relatively folded monomers present in the solution immediately after dilution of the perturbant have very little enzyme activity. During the course of the assembly process, dimers do not accumulate to a significant concentration, indicating that folded monomers associate to form dimers which rapidly associate with other monomers to form trimers.

In summary, it appears that the first step in the assembly of the *C* subunits is the folding of unstructured chains to form inactive (or at most, only partially active) monomers. These monomers then associate to form dimers which rapidly combine with other monomers to form stable, active trimers.

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THERMODYNAMIC STUDIES OF CONCANAVALIN A DIMER-TETRAMER EQUILIBRIA

D. F. Senear and D. C. Teller, *Department of Biochemistry, University of Washington, Seattle, Washington 98105 U.S.A.*

We have studied the reversible dimer-tetramer association equilibria of the jack bean lectin concanavalin A. Equilibrium constants were measured by the high speed sedimentation